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Rabies Virus P Protein Interacts with STAT1 and Inhibits Interferon Signal Transduction Pathways

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Rabies virus P protein is a cofactor of RNA polymerase. We investigated other potential roles of P (CVS strain) by searching for cellular partners using two-hybrid screening. We isolated a cDNA encoding the signal transducer and activator of transcription 1 (STAT1) that is a critical component of interferon type I (IFN-α/β) and type II (IFN-γ) signaling. We confirmed this interaction by glutathione *S*-transferase–pull-down assay. Deletion mutant analysis indicated that the carboxy-terminal part of P interacted with a region containing the DNA-binding domain and the coiled-coil domain of STAT1. The expression of P protein inhibits IFN-α- and IFN-γ-induced transcriptional responses, thus impairing the IFN-induced antiviral state. Mechanistic studies indicate that P protein does not induce STAT1 degradation and does not interfere with STAT1 phosphorylation but prevents IFN-induced STAT1 nuclear accumulation. These results indicate that rabies P protein overcomes the antiviral response of the infected cells.

The interferon (IFN) response is one of the host response's primary defense mechanisms against viral infection. IFNs are classified as α, β, ω, and γ on the basis of their structures and antigenic properties: type I is composed of IFN-α, -β, and -ω and type II of only IFN-γ. IFN-α/β is synthesized and secreted by cells in direct response to specifically viral products, including double-stranded RNA which triggers a cascade of kinase reactions and leads to the activation of specific cellular transcription factors. IFN-α/β is produced by most cells as a direct response to viral infection, while IFN-γ is synthesized almost exclusively by activated NK cells and activated T cells in response to virus-infected cells. Both type I and II IFNs achieve their antiviral effect by binding to their respective receptors (IFN-α/β or IFN-γ receptor), resulting in the activation of a distinct but related "Janus" tyrosine kinase/signal transducer and activator of transcription (Jak/STAT) pathway (17).

Briefly, the interaction of IFN-α/β with its receptors, which consist of IFNAR1 and IFNAR2 molecules, leads to the activation of the "Janus tyrosine kinases" Tyk 2 and Jak1, respectively, via tyrosine phosphorylation. Activated Tyk 2 phosphorylates IFNAR1, which then serves as a binding site for STAT2. STAT2 is then phosphorylated by Tyk 2 on tyrosine 689 and serves as a binding site for STAT1, which in turn is phosphorylated by Jak1 on tyrosine 701. The phosphorylated STATs heterodimerize; the heterodimers dissociate from the receptors and bind to the DNA-binding protein p48 (IFN regulatory factor 9 [IRF-9]) to form the complex IFN-stimulated growth factor 3 (ISGF3). The heterotrimer complexes translocate into the nucleus and bind to the IFN-stimulated response element (ISRE) to induce IFN-stimulated genes (ISGs). The binding of IFN-γ to the IFN-γ receptors IFNGR1 and IFNGR2 leads to the activation of the Janus kinases Jak1 and Jak2, respectively, via tyrosine phosphorylation, which in turn phosphorylate STAT1 on tyrosine. STAT1 homodimers form, migrate to the nucleus, and bind to a DNA element, the gamma-activated sequence termed GAS, to induce specifically the transcription of IFN-γ target genes, such as IRF-1 (17).

All the IFN-induced biological responses are believed to be mediated by ISG products. Among these, the protein kinase double-stranded RNA (PKR), the 2′-5′oligoadenylate synthetase, certain Mx proteins, and promyelocytic protein (PML) have been shown to display intrinsic antiviral activities (2, 9, 40).

Viruses which require cellular machinery for their replication have evolved different strategies to counteract the action of IFN, particularly by altering IFN-signaling and IFN-induced mediators. Several viral proteins acting as IFN antagonists have been identified in *Mononegavirales*, such as members of the family *Paramyxoviridae* (21).

Rabies virus replicates in the host cell cytoplasm. It has a linear, nonsegmented, and single-stranded RNA genome of negative polarity. The ribonucleoprotein contains the RNA genome tightly encapsidated by the viral nucleoprotein (N) and the RNA polymerase complex which consists of the large protein (L) and its cofactor, the phosphoprotein (P). Both L and P are involved in transcription and replication. A positive-stranded leader RNA and five mRNAs are synthesized during transcription. The replication process yields nucleocapsids containing full-length antisense genome RNA, which in turn serves as a template for the synthesis of sense genome RNA.

Like the vesicular stomatitis virus (VSV) P protein, the rabies virus P protein is a noncatalytic cofactor and a regulatory protein: it associates with the L protein in the polymerase complex and interacts with both soluble and genome-associated N proteins. The P protein contains two N protein-binding domains: one domain located in the amino-terminal 177 residues binds to N° (not bound to viral RNA) and the other in the carboxy-terminal region binds to N-RNA (6, 12, 24, 27, 28).

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TABLE 1. Oligonucleotides used for constructs

Construct	Plasmid	Forward oligonucleotide	5′ Site	Backward oligonucleotide	3′ Site
GST-P	pGEX-4T-1	GCCGAATTCAGCAAGATCTTTGTTAAT	EcoRI	GCCCTCGAGGCAGGATGTATAGCG	XhoI
GST-PΔC30	pGEX-4T-1	GCCGAATTCAGCAAGATCTTTGTTAAT	EcoRI	GCCCTCGAGCGCGACCCATCCCAGTAC	XhoI
GST-PΔC75	pGEX-4T-1	GCCGAATTCAGCAAGATCTTTGTTAAT	EcoRI	GCCCTCGAGTATTCCTGAAGATCGGGA	XhoI
GAD-STAT1(140–750)	pGAD GH	GCCGAATTCAGTAAAGTCAGAAATGTG	EcoRI	GCCCTCGAGTTAAAATTCGTGTTTATACTG	XhoI
GAD-STAT1(140–487)	pGAD GH	GCCGAATTCAGTAAAGTCAGAAATGTG	EcoRI	GCCCTCGAGGAAGAAGGACAGATTCCT	XhoI
GAD-STAT1(488–750)	pGAD GH	GCCGAATTCCTGAACCCCCCGTGTGCG	EcoRI	GCCCTCGAGTTAAAATTCGTGTTTATACTG	XhoI
GAD-STAT1(140–315)	pGAD GH	GCCGAATTCAGTAAAGTCAGAAATGTG	EcoRI	GCCCTCGAGGCTCTGAATGAGCTGCTG	XhoI
GAD-STAT1(316–487)	pGAD GH	GCCGAATTCTCCTTTGTGGTGGAACGA	EcoRI	GCCCTCGAGGAAGAAGGACAGATTCCT	XhoI
GAD-STAT1(140–367)	pGAD GH	GCCGAATTCAGTAAAGTCAGAAATGTG	EcoRI	GCCCTCGAGATCTTTGTCAAATGAGAC	XhoI
GAD-STAT1(289–750)	pGAD GH	GCCGAATTCGAGCAGGACCCTATTACA	EcoRI	GCCCTCGAGTTAAAATTCGTGTTTATACTG	XhoI

This domain folds as a single compact domain, as shown from the recently solved crystal structure of the carboxy part of P (28). The major L-binding site resides within the first 19 residues of P (8). The rabies virus P protein is phosphorylated by the following two kinases: the unique cellular protein kinase RVPK (rabies virus protein kinase) and protein kinase C (19). Both kinases phosphorylate specific sites on the P protein, leading to the formation of different phosphorylated forms of the P protein with different motilities in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (19). In addition, the existence of additional shorter P products (P2, P3, P4, and P5) that have different intracellular distributions has been shown (7). The nuclear localization of P3-P5 is due to the presence of a nuclear localization signal (NLS) located in the C-terminal part of the protein, whereas the cytoplasmic distribution of P-P2 is the result of a nuclear export signal located in the N-terminal part of the protein (36). P protein has been shown to interact with the dynein light chain LC8 that could mediate the RNP transport along the neuronal axons (23, 39). The nuclear products of the P gene have been shown to interact with PML nuclear bodies that could be involved in cellular defense mechanisms against viral infection (4).

In order to better understand the role of P during rabies virus cycle, we looked for interacting partners by using the two-hybrid system. STAT1 was isolated as a target of P protein. This interaction required the C-terminal domain of P and the N-terminal half of STAT1. We show here that rabies virus P protein blocks the signal transduction pathway of IFN by preventing IFN-induced STAT1 nuclear translocation.

MATERIALS AND METHODS

Cells and viruses. Human neuroblastoma SK-N-BE cell lines (provided by C. Tuffereau, LVMS, CNRS, Gif sur Yvette, France) were grown in RPMI 1640 plus L-glutamine (Gibco) supplemented with 15% fetal calf serum (FCS). Human glioblastoma astrocytoma U373-MG cells and BSR cells, cloned from BHK 21 (baby hamster kidney), were grown in Dulbecco's modified Eagle medium supplemented with 10% FCS.

The CVS strain of rabies virus was grown in BSR cells.

Stably transfected BSR cells. Stable P-expressing cell lines were produced by cotransfection of BSR cells with plasmid pCDM8, encoding the wild-type P protein previously described (6), and plasmid pSV2 Neo (46) by using lipofectin (Gibco-BRL). After 48 h, the transfection medium was replaced with Dulbecco's modified Eagle medium plus 10% FCS containing Geneticin (500 μg/ml; Sigma). Surviving cells were transferred and expanded. Control BSR cell lines were generated in the same way with pCDM8 and pSV2 Neo. After isolation, stable cell lines were maintained in the presence of 250 μg/ml Geneticin.

Interferons. Human interferon-α (hIFN-α-100) was from Strathmann Biotec, murine interferon αA was from PBL Biomedical Laboratories, (catalog no. 12100-1), and human interferon-γ (specific activity, 2×10^7 U/mg) was from Roussel Uclaf (Romainville, France).

Antibodies. The mouse polyclonal anti-P antibody has been described previously (38). Rabbit anti-STAT1 (catalog no. sc-346), anti-STAT1 phosphotyrosine 701 (catalog no. sc-7988), anti-STAT2 (catalog no. sc-839), anti-PML (catalog no. H-238), and anti-PKR were obtained from Santa Cruz Biotechnology, Inc.

Monoclonal anti-α tubulin from Amersham (catalog no. N356) was used. A rabbit polyclonal anti-VSV prepared in the laboratory was also used.

Plasmid constructions. The *Saccharomyces cerevisiae* L40 yeast expression plasmids pLex10, pGAD, and pLex-lamin were kindly provided by J. Camonis (Institut Curie). The vector pLex10 (pLexA) contains the yeast selectable gene *TRP1* and the LexA DNA-binding domain (BD) (202 residues) coding sequence. The plasmid pGAD contains the yeast selectable gene *LEU2* and the sequence encoding the GAL4 activation domain (AD) (amino acids 768 to 884). These plasmids were used for the expression of encoding fusion proteins.

The constructs pLex-P, pLex-PΔN172, pLex-PΔC75, and pLex-PΔC125 have been described previously (39). Plasmid pLex-PΔC30 differed from the wild-type P gene (CVS strain) by the deletion of 90 nucleotides from the 3′ terminus of the P gene. This deletion was introduced by PCR amplification of the wild-type P gene using oligonucleotides 2HPCA1 and 2HPCB4. 2HPCA1 (GCCGAATTCATGAGCAAGATCTTT) is identical in sequence to the 5′ end of the mRNA and contains an EcoR1 site (underlined) plus GCC. 2HPCB4 (GCC GTCGAC**TTA**TATTCCTGAAGATCG) is complementary to positions 682 to 696 of the PmRNA. Both oligonucleotides are flanked at their 5′ ends with a Sal1 site (underlined) plus GCC preceded by a stop codon (bold). The amplified double-stranded cDNA corresponding to the deleted P gene was digested by EcoR1 and Sal1 and inserted in frame with the LexA-BD into the corresponding cloning sites of pLex10.

The construct pGAL4AD-STAT1 isolated from a yeast two-hybrid screen contains the *Rattus norvegicus* STAT1 gene deleted of the first 420 bp (residues 140 to 750) (BC062079) into pGAD GH (described above). This construct was used as template to obtain different deletion mutants of STAT1 by using PCR with the oligonucleotides described in Table 1. STAT1 with residues 140 to 750 deleted [STAT1(140-750)], STAT1(140-487), STAT1 (140-367), and STAT1 (140-315) differed from the wild-type STAT1 gene by a deletion at the 5′ terminus of 420 bp, 789 bp, 1,149 bp, and 1,305 bp, respectively. STAT1(488-750) and STAT1(289-750) differed from the wild-type STAT1 gene by a deletion at the 3′ terminus of 1,464 bp and 867 bp, respectively. Finally, STAT1(316-487) differed from the wild-type STAT1 gene by a deletion of 948 bp at the 5′ terminus and of 789 bp at the 3′ terminus of the STAT1 gene. The PCR fragments were digested by EcoR1 and Xho1 restriction enzymes and inserted into the corresponding cloning sites of pGAD GH.

The mammalian expression plasmids (pCDM8) encoding M and G proteins have been described previously (4). The plasmids encoding N, P, and the truncated P proteins (PΔC30 and PΔC75), have been described elsewhere (6). The IFN-α-responsive reporter plasmid pISREluc, the IFN-γ-responsive reporter plasmid pGAS-f.luc, and the plasmid pRL-TK have been described elsewhere (10, 14) and were a generous gift of D. Garcin (University of Geneva School of Medicine). Plasmid pISREluc contains four tandem repeats of the IFN-inducible gene 9-27 ISRE fused to the firefly luciferase gene, and pRL-TK, used as a transfection standard, contains the herpes simplex virus thymidine kinase promoter region upstream of the *Renilla* luciferase gene. The plasmid pGAS-f.luc contains IRF1 GAS sequences fused to the firefly luciferase gene.

Plasmid pGST-P was obtained by inserting the P gene into pGEX-4T-1 (Amersham) in frame with the 3′ end of the glutathione *S*-transferase (GST). The P gene was amplified by using PCR with the oligonucleotides described in Table 1. The PCR product was digested by the EcoRI-XhoI restriction enzymes and then inserted into the corresponding cloning sites of pGEX-4T-1. Plasmids pGST-PΔC75 and GST-PΔC30 differed from GST-P by a 225-bp deletion and by

a 90-bp deletion at the 3′ terminus of the P gene, respectively. These deletions were created by PCR amplification of the wild-type P protein using the sets of oligonucleotides shown in Table 1.

Yeast two-hybrid screen. *Saccharomyces cerevisiae* L40 was obtained from J. Camonis. This strain (*MAT***α**, *ade2 leu2 trp1 his3 LYS2::lex op-HIS3 URA3::lex op-LacZ*) carrying *HIS3* and *LacZ* markers under the control of LexA-binding sequences was used for library screening and to assay for protein-protein interaction.

The nerve growth factor-induced PC12 (rat adrenal pheochromocytoma cell line) cDNA library was obtained from J. Camonis. The yeast strain L40, containing the *HIS3* and *LacZ* reporter genes, was transformed with the bait plasmid pLex-P by using a lithium acetate protocol (16, 22). The resulting strain was selected and grown in Trp-deficient medium. A 250-ml culture of pLex-P-expressing L40 in growing phase was transformed with 60 μg of plasmid DNA from the PC12 cDNA library. Double transformants were grown on plates containing medium lacking Trp and Leu (Trp^- Leu^-) to select for the presence of both the bait and library plasmids and deprived of His (Trp^- Leu^- His^-) to select for protein-protein interaction. Positive clones went through two more rounds of selection on Trp^- Leu^- His^- medium, and those that were still able to grow were then assayed for β-galactosidase activity. The $LacZ^+$ His^+ clones were isolated and retransformed with pLex-lamin or pLex-P. The library clones that activated the *LacZ* reporter gene only in the presence of the P gene were sequenced. Homology searches were performed at the National Center for Biotechnology Information (NCBI) using Gapped BLAST and PSI-BLAST (1).

β-Galactosidase assay. The β-galactosidase activity of histidine-positive clones was tested in vivo. In vivo β-galactosidase assays were performed by X-Gal overlay as follows: an X-Gal mixture containing 0.5% agar, 0.1% SDS, 6% dimethylformamide, and 0.04% X-Gal (5-bromo-4-chloro-3-indolyl-βΔ-galactosidase) was overlaid on fresh transformants grown on Trp^- Leu^- dishes, and blue clones were detected after 60 min to 18 h at 30°C. (For the liquid assay, cultures were grown overnight and assayed for β-galactosidase activity with *O*-nitrophenylgalactoside [ONPG] as a substrate [18].)

GST pull-down assay. *Escherichia coli* strain BL21 isolates were transformed with plasmids encoding P or truncated P in fusion with GST and were grown in LB medium until the optical density at 600 nm reached 0.5. Expression was induced for 4 h in the presence of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37°C, and the cells were harvested by centrifugation and resuspended in 100 mM Tris (pH 8), 200 mM NaCl, 1% Triton X-100, and protease inhibitors. After sonication and centrifugation, the cleared lysate was incubated with BSR cell extracts overnight at 4°C. The mixture was immobilized on glutathione agarose beads (Sigma) for 30 min at 4°C. After three washes with PBS, proteins retained on the beads were eluted four times with 250 μl of PBS containing 10 mg ml^{-1} of reduced glutathione (Sigma). Eluted proteins were then analyzed by SDS-PAGE and Western blotting or Coomassie blue staining.

Cell infection and transient transfections. Monolayers of SK-N-BE cells or U373-MG cells were grown in 6-cm dishes to 80% confluence and infected with 5 PFU/cell of rabies virus (CVS strain). Cells were used for experiments at 24 h postinfection.

Monolayers of BSR or IMR5 cells were grown in 12-well plates (from 50 to 80% confluence) and were transfected with 2.5 μg of plasmid DNA by the calcium phosphate coprecipitation procedure. Monolayers of SK-N-BE cells in 12-well plates (at about 50% confluence) were transfected with 1.25 μg DNA plasmid and 2.5 μl lipofectin as described by the manufacturer (Invitrogen).

Luciferase assays. Cells in 12-well plates were transfected with 2.5 μg of plasmid encoding P, PΔN52, or PΔC75, 0.75 μg of pRL-TK, and 2.5 μg of pISREluc (or pGASluc). At 48 h posttransfection, cells were untreated or treated with 2,000 U/ml of murine IFN-α (for BSR cells) or human recombinant IFN-α (or human IFN-γ) (for IMR5 and SK-N-BE cells). Cells were harvested at 6 h after IFN treatment and assayed for firefly and *Renilla* luciferase activity as described by the manufacturer (dual-luciferase reporter assay system; Promega). Relative expression levels were calculated by dividing the firefly luciferase values by those of the *Renilla* luciferase. In some cases, cells transfected with pRL-TK and pISREluc were infected with rabies and treated as described above.

Interferon sensitivity assay and cytopathic effect assay. P-expressing BSR cells and control BSR cells were seeded in 12-well tissue culture dishes with 2×10^5 cells per well and were incubated with the indicated concentrations of murine IFN-α for 24 h. Cells were then infected with VSV (Indiana strain) at a multiplicity of infection (MOI) of 1 PFU/cell. Cell supernatants were harvested at different times, and the virus yields were determined by plaque assay in BSR cells by using the crystal violet method. Cell lysates were analyzed by immunoblotting with anti-VSV serum to detect the presence of viral proteins.

Cell extracts and immunoblotting. Cells were washed with PBS and lysed on ice in 1 ml of buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 0.5% NP-40 in the presence of 10 μM sodium orthovanadate and an antiprotease cocktail (2 μg of leupeptin per ml, 2 μg of antipain per ml, 2 μg pepstatin per ml, 2 μg of chymostatin per ml, 16 μg of aprotinin per ml). Lysates were centrifuged at 13,000 × *g* for 2 min. Supernatants constitute cytoplasmic extracts, and pellets were resuspended with 20 mM HEPES (pH 7.9), 420 mM KCl, 20% glycerol, 1 mM EDTA, 2 mM 2-mercaptoethanol, 10 μM sodium orthovanadate, and antiprotease cocktail in order to extract nuclear proteins (29).

In some experiments, cells were washed and resuspended in PBS, lysed in hot Laemmli sample buffer, and boiled for 5 min. Proteins were analyzed on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The proteins were blocked on the membranes with 10% skim milk in Tris-buffered saline for 2 h and incubated overnight at 4°C with different antibodies according to the experiments. The blots were then washed extensively in Tris-buffered saline-Tween and incubated for 1 h with the appropriate peroxidase-coupled secondary antibodies (Sigma). All of the blots were revealed by chemiluminescence (ECL; Amersham).

Immunoprecipitation. Twenty-four hours after infection, cells were harvested by scraping into cold PBS and lysed on ice in 1 ml of buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40, and the antiprotease cocktail described above. Nuclei were eliminated from the lysate by centrifugation at 12,000 × *g* for 10 min at 4°C. The cytoplasmic fractions were incubated overnight at 4°C with the anti-STAT2 antibody. Immune complexes were precipitated by incubation with protein A-Sepharose for 1 h at 4°C, washed three times, and denatured in the Laemmli disruption buffer. Immunoprecipitated proteins were analyzed by Western immunoblotting using the rabbit anti-STAT1 or anti-pSTAT1 antibody as described above.

Immunofluorescence staining and confocal microscopy. Cells were fixed and permeabilized for 5 min with methanol at −20°C. They were then prepared for double-immunofluorescence staining and analyzed by confocal microscopy. The intracellular distribution of STAT1 or phosphorylated STAT1 was analyzed by using the rabbit anti-STAT1 or anti-pSTAT1 antibody at a dilution of 1/100 or 1/50, respectively, and the corresponding anti-rabbit immunoglobulin G antibody conjugated to Alexa Fluor 568 (Molecular Probes). The viral P protein was stained using mouse polyclonal anti-P at a dilution of 1/1,000, and the corresponding anti-mouse immunoglobulin G antibody conjugated to Alexa 488 (Molecular Probes).

Confocal laser microscopy was performed on a Leica SP2 microscope (40× oil-immersion objective) using blue laser excitation at 488 nm (Alexa 488; green fluorescent protein) and/or green laser excitation at 545 nm (Alexa 568) in sequential recording mode.

RESULTS

Identification of Stat1 as a cellular partner of the rabies virus P protein by yeast two-hybrid system. In order to identify rabies virus P protein-interactive cellular factors, we performed a two-hybrid screen by using the P fused to the DNA-BD of LexA as a bait against a cDNA library of PC12 cells (induced by nerve growth factor), in which each DNA was fused to the sequence encoding the GAL4 AD. The yeast L40 strain containing the two LexA-responsive reporter genes, *HIS3* and *lacZ*, was used to assay the interactions. Seventy clones of the 4×10^5 independent transformants were selected on the basis of their ability to activate the transcription of both *HIS3* and *lacZ* reporter genes. One-half of the clones were analyzed further after the elimination of false positives and were partially sequenced. Two independent clones were found to encode the protein STAT1 lacking the NH2 terminal domain (Stat1 ΔN139 from residues 140 to 750).

The yeast L40 strain was cotransformed with the DNA of one positive clone and either the BD-P-encoding plasmid or the BD-lamin-encoding plasmid. Both the *HIS3* and *LacZ* reporter genes were activated if both P and STAT1 were coexpressed (Fig. 1A). Under conditions in which no P-STAT1 interaction could take place (coexpression of BD and AD, BD-lamin and AD-STAT1, or BD-P and AD), the reporter genes were not induced, resulting in no growth of yeasts in

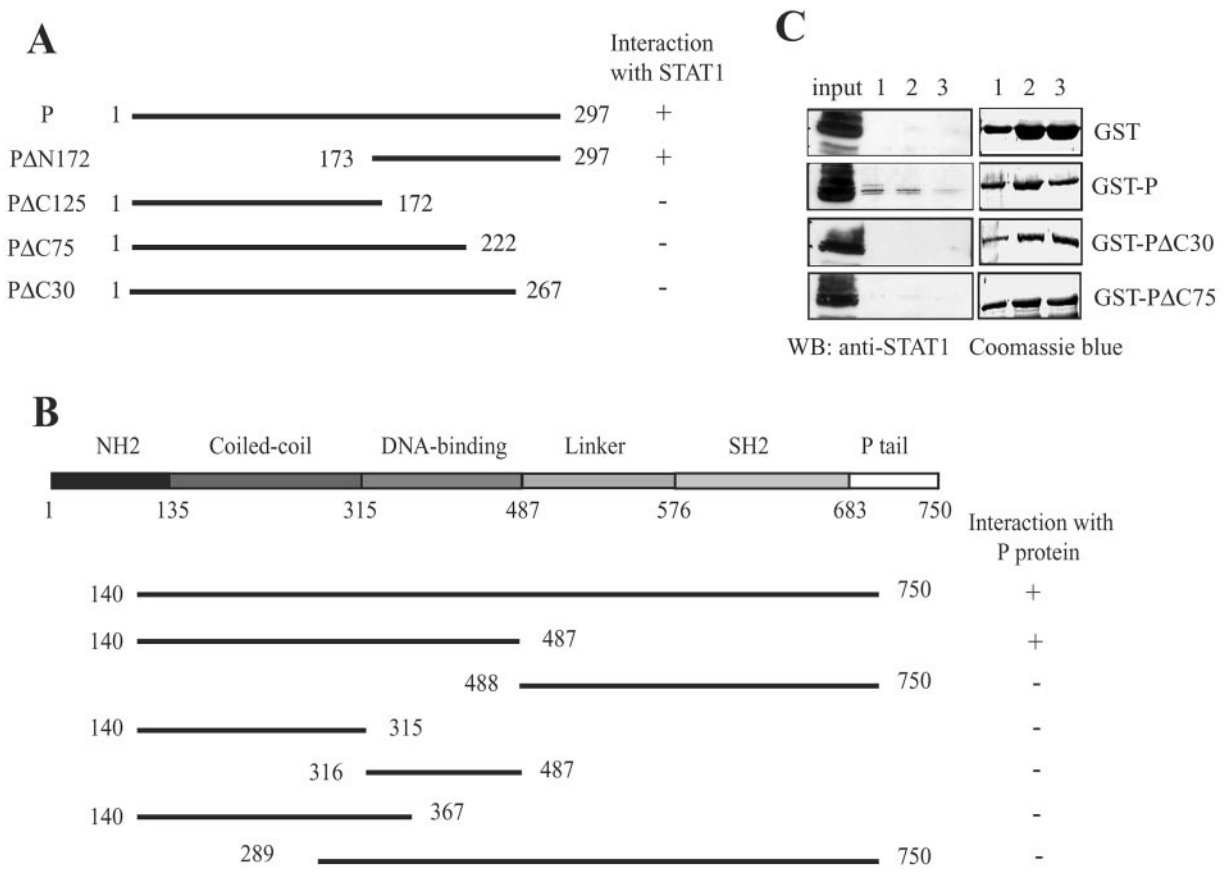


FIG. 1. Mapping of P and STAT1 interacting domains. (A) Identification of the STAT1-binding domain on P by a two-hybrid system. Expression vectors encoding P or truncated P proteins fused to the DNA-binding domain of LexA were transformed into L40 yeast together with the construct encoding STAT1ΔN140 from *Rattus norvegicus* fused to the GAL4-activating domain. Cells were streaked onto plates containing medium lacking tryptophan and leucine (Trp^- Leu^-) for double transformants or lacking tryptophan, leucine, and histidine (Trp^- Leu^- His^-) to assay the activation of the *HIS3* reporter gene. The induction of the LacZ reporter gene was assayed by the appearance of blue colonies as described in Material and Methods. +, Presence of blue yeast cells; −, no blue yeast cells. (B) Identification of the P-binding domain on STAT1. Expression vectors encoding various truncated STAT1 fused to the GAL4-activating domain were transformed into L40 cells together with the plasmid encoding the full-length P protein fused to the DNA-binding domain of LexA. Protein interactions were tested as described in panel A. (C) GST pull-down results. BSR cell extracts containing endogenous STAT1 were incubated with BL21 extracts containing GST alone, GST-P, GST-PΔC30, or GST-PΔC75 for 16 h at 4°C. Each mixture (input) was glutathione-agarose immobilized. Agarose-bound proteins were eluted three times (1, 2, 3) with PBS containing reduced glutathione and were analyzed by immunoblotting with anti-STAT1 antibody. Proteins were also stained with Coomassie brilliant blue to visualize the amount of agarose-immobilized GST proteins. WB, Western blot.

medium lacking histidine or white colonies in the presence of X-Gal (not shown).

Identification of P-STAT1 interacting domains. To identify the domain of P that mediates binding to STAT1, N-terminal-truncated and C-terminal-truncated P proteins with coding sequences fused to the coding sequence of LexA BD were generated (Fig. 1A). The partial STAT cDNA (GAL4AD-Stat1ΔN139) originally isolated from the yeast two-hybrid screening was used for these studies. Results were obtained by assessing the ability of the yeast to grow in the absence of histidine, shown by the appearance of blue colonies in the presence of X-Gal (Fig. 1A). The amino-terminal-truncated P protein PΔN172 very efficiently activated the transcription of the *HIS3* and *LacZ* genes and thus interacted with Stat1. In contrast, the C-terminal deletion of 125 amino acids impaired binding to STAT1, indicating that the binding domain was located in the C-terminal part of P. To further define the precise binding domain, PΔC75 and PΔC30 were tested for their abilities to bind to STAT1. None of these deleted mutants interacted with Stat1 (Fig. 1A). This indicated that the STAT1-binding domain of P was located in the C-terminal domain of P and that the last 30 residues between 268 and 297 were essential for the binding site to be functional.

STAT1 protein contains six domains. The N domain missing in the isolated two-hybrid clone is followed by a coiled-coil domain (residues 140 to 317) implicated in protein-protein interaction, a DNA-binding domain (residues 318 to 487), a linker domain (residues 488 to 576) that participates in DNA binding, an SH2 domain that mediates dimerization and receptor binding, and a C-terminal transactivation domain (700 to 708) (Fig. 1B). To determine the regions of STAT1 that interact with P protein, the N-terminal (140 to 487) or C-terminal (488 to 750) part of STAT1 fused to the GAL4 AD was coexpressed with Lex-P in yeast. The N-terminal domain from residues 142 to 487 bound P (Fig. 1B). To determine more precisely which domain between the coiled-coil domain

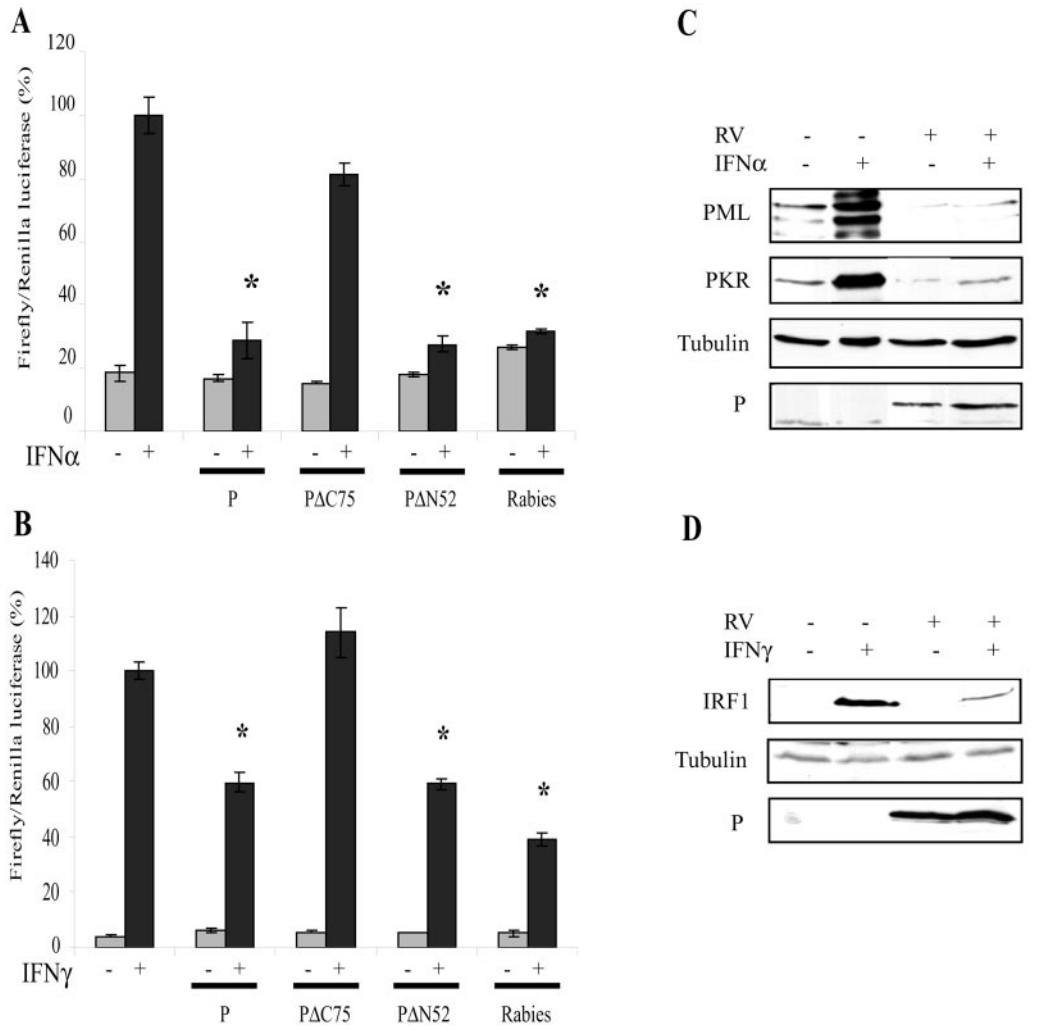
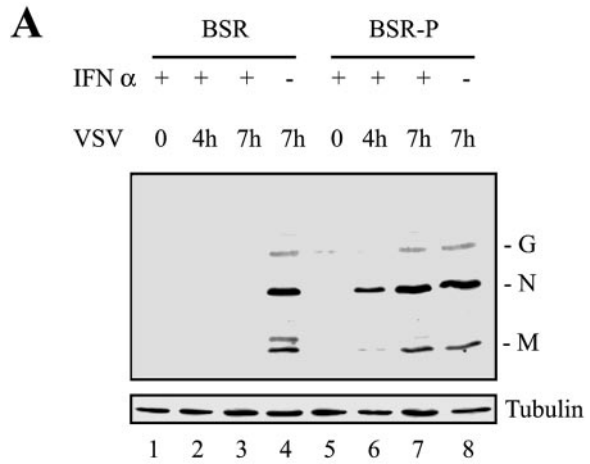


FIG. 2. Expression of the rabies virus P protein inhibits IFN-α and IFN-γ signaling. (A) Human neuroblastoma SK-N-BE cells were transfected with ISRE-firefly luciferase reporter plasmid (pISRE-f.luc) and *Renilla* luciferase expression vector (pTK-r luc) and either empty vector or plasmid expressing P, PΔN52, or PΔC75 as indicated. At 24 h after transfection, cells were treated with 2,000 U/ml of human IFN-α (+) for 6 h prior to lysis and luciferase assays or left untreated (−). Neuroblastoma SK-N-BE cells were transfected with pISRE-f.luc and pTK-r luc and then infected (rabies) or not with 10 PFU/cell of rabies virus at 24 h after transfection. Cells were IFN treated at 48 h posttransfection as described above. All bars represent average values of firefly luciferase from triplicate samples, normalized to the expression of *Renilla* luciferase and expressed as percentages of IFN-stimulated controls; error bars indicate standard deviations. Bars which are statistically different from the corresponding control ($P < 0.001$) are labeled by asterisks. (B) Same as in panel A but using an IFN-γ-responsive GAS luciferase reporter gene instead of ISRE luciferase. (C) SK-N-BE cells were uninfected (−) or infected (+) with rabies virus (RV) for 24 h and were then treated with 2,000 U of human IFN-α/ml for 24 h. The expression of PKR and PML was studied by Western blot analysis with specific antibodies. (D) The same experiment was performed with 2,000 U/ml of human IFN-γ and the expression of IRF1 was analyzed by Western blot analysis..

or the DNA-binding domain was involved in the interaction with P, additional deletions of the N-terminal domain of STAT1(140-315), STAT1(140-367), and STAT1(316-487) were generated. These amino-terminal-truncated STAT1 proteins failed to interact with P. The lack of interaction could be due to the high sensitivity of STAT1 to deletion, which may alter the folding of the protein. This result indicates that the P-binding domain is located in the coiled-coil and DNA-binding domain.

We next performed in vitro GST pull-down assays to confirm the yeast two-hybrid interaction results. BL21 extracts containing GST, GST-P, GST-PΔC30, or GST-PΔC75 fusion protein were incubated with BSR cell extracts containing endogenous STAT1. The mixtures of glutathione-agarose-immobilized and agarose-bound proteins were eluted and analyzed by immunoblotting with anti-Stat1 antibody (Fig. 1C). GST-P interacted with both α and β isoforms of STAT1 (Fig. 1C, left panel). This interaction was impaired by the carboxy-terminal truncation of P (Fig. 1C, right panel). These results confirm that the STAT1-binding domain is located in the carboxy domain of P.

Rabies virus P protein inhibits IFN-α and IFN-γ transcriptional responses. To test the potential of P to function as an IFN antagonist, IFN-α/β and IFN-γ luciferase reporter gene assays were conducted in human neuroblastoma SK-N-BE cells in the presence or absence of expressed P protein. IFN-α treatment resulted in the induction of ISRE-luciferase re-



B

	Untreated	IFN α
BSR	7×10^8	10^6
BSR-P	$1{,}6\times10^9$	$1{,}2\times10^9$

FIG. 3. Effect of P protein on antiviral response. (A) A control BSR clone and a clone of P-expressing BSR cells were treated with 2,000 U/ml IFN-α and then infected for various times as indicated with VSV at a MOI of 1 PFU/cell. Viral proteins were detected by Western blot analysis with a rabbit anti-VSV. +, IFN treated; −, not IFN treated. (B) Virus yields of the supernatant of cells infected for 7 h were obtained by plaque assay on BSR cells (expressed as PFU/ml).

porter gene activity of about fivefold relative to untreated cells, reflecting the actions of the endogenous ISGF3 transcription complex (Fig. 2A). The expression of the rabies virus P protein inhibited IFN-α-responsive transcription, as did the amino-terminal-truncated PΔN52 protein. As expected, the truncated PΔC75 protein that fails to interact with STAT1 did not interfere with the ability of IFN-α to induce a transcriptional response, indicating that the IFN evasion activity correlates with the role of the C-terminal domain in the interaction of P with STAT1.

The response to IFN-γ was tested using a STAT1-dependent IFN-γ activation sequence GAS-luciferase reporter gene (Fig. 2B). Similarly, IFN-γ treatment enhanced the firefly luciferase activity about eightfold relative to untreated cells and P protein-dependent inhibition was reproducibly observed but at a lower level than that in the case of IFN-α.

IFN-α and IFN-γ responses were also studied in the context of viral infection. Human neuroblastoma SK-N-BE cells were transfected with reporter luciferase plasmids and then infected 24 h later. Rabies infection affected luciferase expression induced by both IFNs (IFN-α/β and IFN-γ), suggesting that the effect of P on IFN response existed also during viral infection and, thus, was not altered by the presence of other viral proteins (Fig. 2A and B). The same IFN-α-signaling inhibition by P protein was observed in human glioblastoma U373-MG cells and in hamster BSR cells (data not shown), indicating that rabies virus P protein is an effective inhibitor of IFN signaling in both human and hamster systems. In order to exclude the involvement of another viral protein(s) on the inhibition of IFN transcriptional responses, SK-N-BE cells were transfected

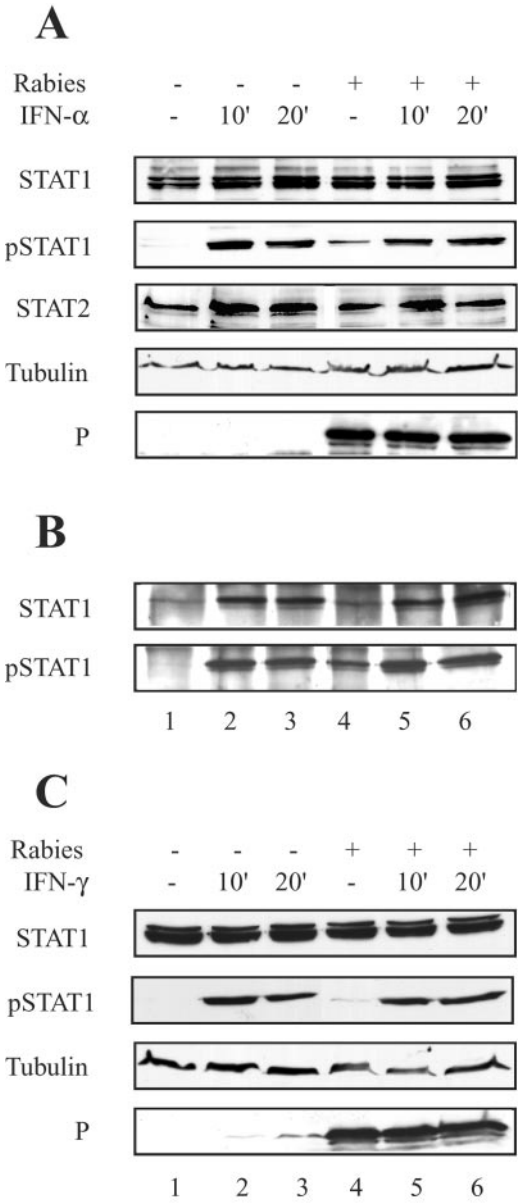


FIG. 4. Effect of rabies virus infection on STAT tyrosine phosphorylation and dimerization. SK-N-BE cells were infected with 10 PFU per cell of rabies virus (+, lanes 4, 5, and 6) or not infected (−, lanes 1, 2, and 3). Twenty hours after infection, cells were treated with 2,000 U/ml of human IFN-α for 10 min (lanes 2 and 5) or 20 min (lanes 3 and 6) or left untreated (−, lanes 1 and 4). (A) Cell lysates were analyzed directly by immunoblotting (SDS-12% PAGE) with anti-STAT1, anti-pSTAT1 (phosphorylated tyrosine 701), anti-STAT2, antitubulin, or anti-P antibodies. (B) Cell extracts were immunoprecipitated with anti-STAT2 antibody, and the immuncomplexes were then analyzed by immunoblotting with anti-STAT1 or anti-pSTAT1 antibodies. (C) Same as in panel A but using IFN-γ instead of IFN-α.

with the rabies N, M, or G gene and the luciferase expression was tested in the presence of each protein. No inhibition of luciferase expression was observed in these conditions (data not shown).

Since the rabies virus infection or P protein interferes with IFN signaling, we studied whether the expression of some ISG

products, such as PKR and PML, would also be reduced by P protein expression in infected cells. Cells infected for 24 h were then treated by IFN-α for 24 h, and the expression of PKR and PML was studied by Western blot analysis (Fig. 2C). As expected, PML and PKR expression was induced by IFN-α in uninfected cells. Rabies virus infection before IFN treatment abolished the capacity of the cytokine to induce the expression of both PKR and PML (Fig. 2C). In order to test the effect of rabies infection on IFN-γ signaling, the expression of IRF1 that is specifically induced by IFN-γ was analyzed by Western blot. Again, rabies virus infection before IFN-γ treatment impaired the induction of IRF1 expression by this cytokine (Fig. 2D). Together, these results confirmed that P protein functions as an inhibitor of both IFN-α and IFN-γ transcriptional responses.

P protein antagonizes the antiviral effect of IFN-α. To investigate the effect of P protein on the establishment of an antiviral state, we used BSR cell lines expressing the P protein and we compared the protective effect of IFN-α on infection by VSV, a known IFN-sensitive virus, in a control BSR-cell clone and in two clones of P-expressing BSR cells. Cells were incubated with 2,000 units/ml IFN-α and then infected for various times with VSV at a MOI of 1 PFU/cell. Cell supernatants were harvested, and viral titers were assayed by plaque formation on BSR cells. Viral proteins (G, N, and M) were detected by Western blot analysis of cell extracts (Fig. 3). As expected, IFN-α inhibited VSV replication in control BSR cells (Fig. 3). In contrast, VSV protein amounts were unaffected by IFN treatment in the presence of P (Fig. 3A). This is confirmed by VSV titration in both cell lines (Fig. 3B). These results indicated that rabies P protein counteracts the antiviral effect of IFN.

Effect of P protein on STAT1 tyrosine phosphorylation and dimerization. Since the degradation of STAT1 has been shown to contribute to the blockade of IFN signaling, we first analyzed whether P protein alters STAT1 expression in infected cells. Immunoblotting was performed using antibody specific for STAT1. Total amounts of STAT1 α/β between uninfected and infected cells were almost the same (Fig. 4A). This indicates that P protein does not massively target STAT1 for degradation.

We then examined the early steps of IFN-α signaling, STAT1 protein-activating tyrosine phosphorylation and STAT1-STAT2 heterodimerization. The effect of P protein on tyrosine phosphorylation of STAT1 was examined (Fig. 4A). Uninfected cells or infected cells were untreated or treated with IFN-α and then lysed. Immunoblotting with phosphotyrosine-specific STAT1 antiserum revealed that phosphorylated STAT1 (pSTAT1) α/β increased within 10 min of IFN in uninfected cells (Fig. 4A, lanes 1, 2, and 3) as expected and that this level was unchanged in infected cell extracts (Fig. 4A, lanes 4, 5, and 6). The presence of low amount of activated STAT1 in untreated infected cells (Fig. 4A, lane 4) should be noted. This could be due to the secretion of IFN after 24 h of rabies infection. The same activation of STAT1 was observed in uninfected and infected cells in the presence of IFN-γ (Fig. 4C, lanes 2, 3, 5, and 6).

The effect of P on STAT1-STAT2 heterodimerization was then analyzed by a coimmunoprecipitation assay (Fig. 4B). Lysates of cells untreated or treated with IFN-α were immunoprecipitated with STAT2-specific antiserum, and immune complexes were processed for STAT1 immunoblotting. In both uninfected and infected cells, STAT1 was detected in the STAT2 immune complexes following IFN stimulation (Fig. 4B, lanes 2, 3, 5, and 6). Immunoblotting with the STAT1 phosphotyrosine-specific antiserum confirmed that activated pSTAT1 was present in the immunoprecipitates. This result indicates that rabies virus infection does not interfere with STAT1 phosphorylation or STAT1-STAT2 dimerization.

Rabies P protein prevents IFN-induced STAT1 nuclear accumulation. The IFN-α activated ISGF3 transcription complex containing tyrosine-phosphorylated STAT1 and STAT2 associated with IRF-9 (p48) rapidly translocates to the nucleus upon IFN treatment. Indirect immunofluorescence was used to analyze the effect of P or rabies infection on IFN-α-induced STAT1 nuclear translocation. Cells were transfected with plasmid encoding P and stained with anti-P antibody and with anti-STAT1 (Fig. 5A) or anti-pSTAT1 antibodies (Fig. 5B and C). STAT1 was mostly cytoplasmic in unstimulated cells and rapidly redistributed to the nucleus following IFN-α stimulation (Fig. 5A). The expression of rabies P protein in the cell prevented the STAT1 nuclear accumulation in response to IFN-α, resulting in a cytoplasmic distribution of STAT1 (Fig. 5A). As expected, the nuclear form of STAT1 after IFN-α treatment was phosphorylated (data not shown). When P protein was expressed, no pSTAT1 was detected in the nucleus (Fig. 5B, upper panel) and it was possible to observe pSTAT1 accumulation in the cytoplasm (Fig. 5C). In contrast, cells expressing PΔC75 had activated pSTAT1 in the nucleus (Fig. 5B, upper panel).

Phosphorylated STAT1 homodimers are the active complex formed in response to IFN-γ, and this STAT1 homodimer rapidly translocates to the nucleus to activate the IFN-γ-responsive gene. The expression of P protein and not of PΔC75 also efficiently prevented nuclear accumulation of pSTAT following IFN-γ stimulation (Fig. 5B, lower panel, and C).

Intracellular distribution of STAT1 and pSTAT1 was also analyzed in the context of rabies infection. While IFN-α and -γ treatment resulted in efficient STAT1 nuclear translocation in uninfected cells, no STAT1 protein nuclear accumulation was observed in infected cells in agreement with the results obtained with isolated P protein expression (Fig. 5A and B). This was confirmed by cellular fractionation (data not shown). These results are consistent with the observed suppression of IFN-α and -γ signaling. These findings indicate that the mechanistic basis for rabies virus P protein suppression of IFN-α and -γ signaling is the result of prohibiting pSTAT1 nuclear accumulation.

DISCUSSION

In this study, we have shown that rabies virus P protein plays an important biological function in targeting type I and type II IFN-signaling pathways and inhibiting the IFN-induced responses.

We identified the target protein of rabies P protein as STAT1 by using a two-hybrid screening system. STAT1 is a key transcription factor in the IFN-signal transduction pathways that leads to cellular antiviral and immunomodulatory responses. The STAT1-binding site is located in the C-terminal part of P and requires the last 30 residues to be functional. The

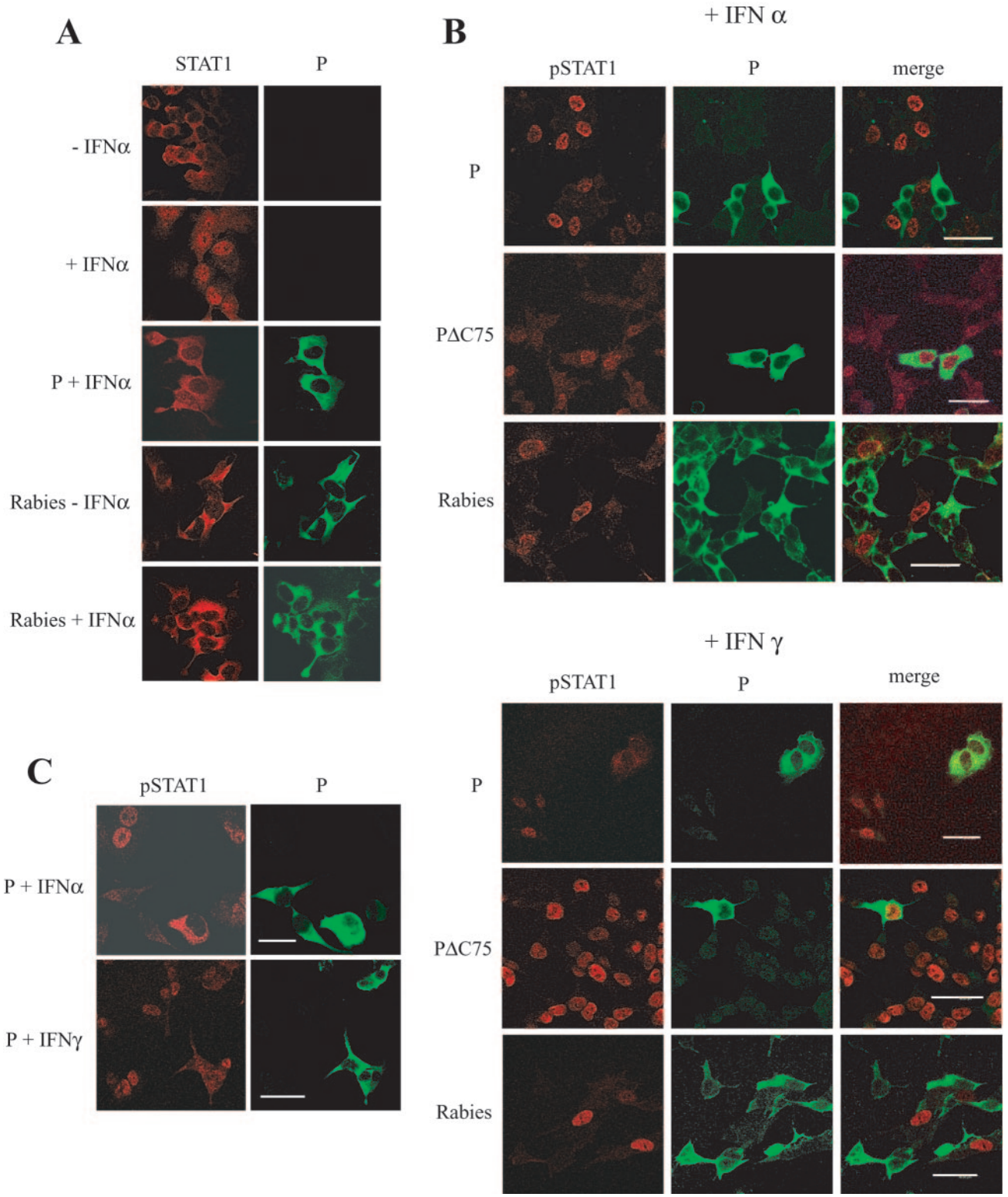


FIG. 5. Rabies virus infection and P protein expression prevent IFN-α and IFN-γ induced STAT1 nuclear accumulation. (A) Human neuroblastoma SK-N-BE cell lines were uninfected or infected with rabies virus or transfected with plasmids expressing P or PΔC75 as indicated. Forty-eight hours after transfection or 24 h after infection, cells were unstimulated or stimulated with 2,000 U/ml of hIFN-α for 25 min and they were fixed, permeabilized, and then stained with anti-P and anti-STAT1 antibodies. (B) SK-N-BE cells were transfected with plasmids expressing P or PΔC75 or were infected with rabies virus and treated with 2,000 U/ml of hIFN-α (upper panel) or of hIFN-γ (lower panel). Cells were then stained with anti-P and anti-pSTAT1 antibodies. (C) SK-N-BE cells were transfected with plasmids expressing P and treated with 2,000 U/ml of hIFN-α or of hIFN-γ. Cells were then stained with anti-P and anti-pSTAT1 antibodies. The scale bars correspond to 40 μM.

P-binding site could not be precisely defined on STAT1: it is contained in the coiled-coil domain and the DNA-binding domain. Due to the structural features of STAT1, deletions of one of these domains may lead to the incorrect folding of the protein. Coiled-coil domain has been implicated in protein-protein interactions, and sequences of both the coiled-coil domain and the DNA-binding domain have been shown to be involved in nuclear import of STAT (11, 26, 31).

The expression of P protein alone inhibits both IFN-α and IFN-γ transcriptional responses in different cellular contexts. This inhibition is correlated with the direct interaction of P with STAT1 since a P mutant lacking the STAT1-binding site does not inhibit the IFN transduction signal. This inhibition effect is also detected in rabies virus-infected cells, indicating that P-STAT1 interaction was not affected by P-N and P-L interactions during viral infection although the N binding has also been located in the C terminus part of P (6, 12). Moreover, we have shown that P counteracts the resulting antiviral effect induced by IFN-α by using a cell line stably expressing P protein and an indicator virus, such as VSV.

Our studies have revealed that rabies virus P protein does not induce down-regulation of STAT1 nor prevent IFN-induced STAT1 protein-activating tyrosine phosphorylation but blocks STAT1 nuclear accumulation following IFN-α or IFN-γ treatment.

Inhibitors of the IFN-signaling pathway have been previously identified in other viral families with DNA or negative-strand RNA genomes. The paramyxoviruses have developed the ability to efficiently inactivate STAT protein function in many cases by using a phosphoprotein P gene product (21). Among paramyxovirus species, there is a great diversity in STAT-signaling evasion. *Rubulavirus* V proteins (SV5 and mumps) block IFN by inducing STAT1 degradation (35, 48). In the genus *Respirovirus*, the Sendai virus C protein induces STAT1 degradation and inhibits STAT1 phosphorylation (15, 25). Measles virus, a prototype of the *Morbillivirus* genus, encodes the V protein, which does not degrade STATs or prevent STAT phosphorylation but blocks IFN-induced STAT1 and STAT2 nuclear import (34, 47). In addition, the V protein of the Nipah virus and Hendra viruses from the genus *Henipavirus* blocks IFN-α/β and IFN-γ by preventing STAT1 phosphorylation and STAT1 nuclear accumulation (42, 43). Recent studies have shown that *Henipavirus* V proteins target STAT proteins by inducing the formation of high-molecular-weight STAT-containing complexes localized in the cytoplasm. This sequestration of STAT1 and STAT2 prevents STAT activation and blocks IFN-induced antiviral response.

In the case of rabies virus, P inhibits nuclear translocation of phosphorylated STAT1 homodimers as well as STAT1/STAT2 heterodimers after stimulation with both IFN-γ and IFN-α. Upon exposure to cytokines, phosphorylated STATs dimerize via SH2 domain interactions, leading to their accumulation in the nucleus. Although the mechanism is unclear, it has been proposed that nuclear accumulation of STAT1 depends on nuclear import and nuclear retention of STAT1. Nuclear import requires STAT dimerization that allows exposure of a nonconventional importin-dependent NLS within the DNA-binding domain. This NLS is recognized by importin α5 (11, 29). Additional residues in the coiled-coil domain seem to also contribute to nuclear import of some STAT (26). STAT1 nuclear retention depends on the integrity of the DNA-binding domain. Indeed, DNA-bound STAT1 is protected from dephosphorylation and is thus transiently retained in the nucleus (32, 33). Loss of DNA binding is associated with the loss of nuclear retention (30). Interaction of rabies virus P protein with the coiled-coil or DNA-binding domains of STAT1 could interfere with both the nuclear import machinery and DNA-binding function. Furthermore, we have previously shown that rabies P protein contains a nuclear export signal (36) which could be involved in the nuclear export of STAT1. Further studies are under investigation to know the molecular mechanism involved in the inhibition of STAT1 nuclear translocation.

A recent report shows that rabies P protein prevents the transcription of IFN-β in infected cells by interfering with the phosphorylation of interferon regulatory factor 3 (5). Our data provide evidence that rabies virus P protein inhibits IFN-induced biological responses by impairing the nuclear translocation of STAT1. Interestingly, rabies P protein interacts directly with the IFN-induced PML, leading to a reorganization of PML nuclear bodies (4). All these data together indicate that rabies P protein mediates the inhibition of different IFN pathways: it inhibits IFN production and IFN signaling and may impair antiviral activity of IFN-induced proteins. Several viruses are known to target multiple IFN pathways in order to maximize their ability to evade the innate immune response, and these effects are mediated by the same viral proteins (for a review, see references 3 and 13). In the case of paramyxoviruses, the V protein of SV5 has been shown to inhibit both IFN signaling (10) and IFN production (20, 37). Similarly, the Henipavirus virus V and W proteins both function as inhibitors of JAK/STAT signaling (41, 42, 43, 44) and of the production of IFN (45).

In conclusion, rabies virus P protein is a multifunctional protein that, in addition to its role in viral RNA synthesis, acts as an interferon antagonist and counteracts the host's innate immune response.

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